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Marine alkaloid oroidin analogues with antiviral potential: a novel class of synthetic compounds targeting the cellular chaperone Hsp90

Short running title: Oroidin analogues with antiviral potential

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Keywords: Hsp90, oroidin, clathrodin, hepatitis C virus, Chikungunya virus, replicon model

Abstract

Marine organisms and their metabolites are a diverse source of scaffolds for potential pharmacological molecular probes and, less frequently, for pharmaceutical lead compounds. In this study, 157 synthetic analogues of marine sponge-derived alkaloids clathrodin and oroidin were screened against replicon models of two RNA viruses, hepatitis C (HCV) and Chikungunya virus (CHIKV) as part of a larger screening project. Four compounds were found to selectively inhibit the HCV replicon (IC_{50} 1.6-4.6 μM). These belong to the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole class of compounds originally designed to target the ATP-binding site of bacterial DNA gyrase. The ATP-binding site of this bacterial protein has high structural similarity to the ATP-binding site of heat shock protein 90 (Hsp90), a host-cell chaperone universally required for viral replication, which led us to examine inhibition of Hsp90 as the compounds' potential mechanism of action. Binding of the four hit compounds to Hsp90 was evaluated through microscale thermophoresis and molecular modelling, which supported our hypothesis of interaction with Hsp90 (K_d 18-79 μM) as basis for the compounds' antiviral activity. The presented novel structural class of small molecules that target the Hsp90 ATP-binding site has excellent potential for further antiviral drug development because of the compounds' low toxicity and synthetic accessibility.

1. Introduction

Marine sponges are a rich source of natural products possessing a variety of biological activities.[1] The chemical versatility of sponges has provided numerous developmental pharmaceuticals.[2] Due to challenges in collection and cultivation of marine sponge species, chemical synthesis extends the possibilities to study the bioactive properties of sponge-derived compounds and their derivatives. Clathrodin and oroidin are pyrrole-2-aminoimidazole alkaloids isolated from sponges of the genus *Agelas*, and were initially shown to display voltage-gated sodium channel modulatory activity[3] and inhibition of bacterial biofilm formation[4], respectively. Recently, we have designed and synthesized several structural series of conformationally restricted oroidin analogues and reported their biological activities including voltage-gated sodium channel modulatory activity[5-8], inhibition of bacterial biofilm formation[9], induction of apoptosis in THP-1 and HepG2 cell lines[10], DNA gyrase inhibition[11-12] and antimicrobial activity[13].

This study encompassed the biological evaluation of 157 synthetic clathrodin and oroidin analogues for their antiviral activity. All compounds were initially screened in replicon models of two RNA viruses, Hepatitis C virus (HCV; genus *Hepacivirus*) and Chikungunya virus (CHIKV; genus *Alphavirus*). HCV is a small, enveloped, positive-stranded (+)-RNA virus that causes severe liver disease, a global public health problem estimated to cause 500 000 deaths annually.[14] HCV therapy relied for long on the use of interferons together with the guanosine analogue ribavirin. In recent years, introduction of direct-acting antivirals has brought notable progress to the clinical treatment of HCV infections.[15]

Despite recent therapeutic advances, the treatment costs are extremely high and novel therapies are therefore out of reach for most HCV patients even in high-income countries.[16] Chikungunya virus is spread by *Aedes* mosquitoes and it is the cause of Chikungunya fever.[17] This disease causes acute high fever, polyarthralgia, myalgia, nausea, headache and skin symptoms, as well as relapsing chronic rheumatic manifestations. There are currently no specific treatments or vaccines against CHIKV.

The aim of the primary evaluation of the compound library was finding compounds with potential to suppress viral replication. The use of replicon cell models enables identification of potential suppressors of viral replication in an efficient manner and without the need for biosafety level 3 (BSL-3) handling. Using models of two different RNA-viruses in the first study phase made it possible to identify compounds that target non-viral proteins involved in the replication cycle in addition to compounds that have selectivity between the models. Based on the primary evaluation in the HCV and CHIKV models, four hit compounds **1-4,** all of which belong to the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole structural class of oroidin analogues, were selected and subjected to further evaluation. The objectives of these follow-up studies were to determine the compounds' efficacy, specificity and antiviral mechanism of action.

2. Methods and materials

2.1. Design of 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole structural class of oroidin analogues

Since marine alkaloids clathrodin, hymenidin and oroidin (Fig. 1) possess a potentially unstable double bond between the pyrrolamide and 2-aminoimidazole moieties[18], we designed and synthesized several libraries of their conformationally restricted analogues.[10] Among them we prepared a library of

4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazol-2-amine derivatives in which the imidazole ring was isosterically replaced by the thiazole ring (Fig. 1, class **I**; Supplementary table S1, compounds **2**, **5-11**).[6] Recently, compounds **2** and **11** (Fig. 1 and Supplementary table S1) were identified as *Escherichia coli* DNA gyrase inhibitors and were subjected to structure-based optimization which resulted in potent DNA gyrase inhibition (Fig. 1, class **II**; Supplementary table SI, compounds **1**, **3**, **4**, **12-38**).[11] The above mentioned screening library of 157 oroidin analogues contained 38 compounds of this structural type.

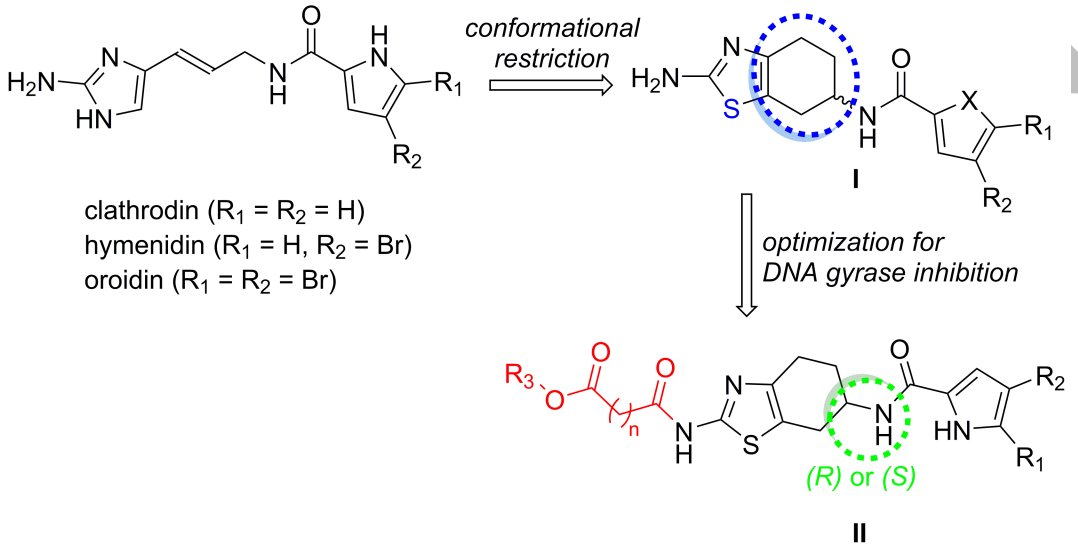

Fig. 1. Design of conformationally restricted oroidin analogues and their optimization toward potent DNA gyrase inhibitors.

2.2. Screening of compounds in Chikungunya virus (CHIKV) and hepatitis C virus (HCV) replicon cells

A library of 157 synthetic analogues based on the lead structures of marine alkaloids clathrodin and oroidin was subjected to primary screening in both HCV and CHIKV replicon cell models. In the primary screening phase, all compounds were tested in triplicate at a concentration of 50 μM. The

activity threshold in the primary screening phase was set at >50% inhibition of replicon expression for both applied models.

2.3. Cell culture

The Huh-7 cell line expressing a HCV replicon was kindly provided by Prof. Ralf Bartenschlager (University of Heidelberg, DE) and has been described by Vrojlik and co-workers (2003).[19] Under continuous G418 selection, the cell line expresses a subgenomic replicon of the HCV genotype 1b genome and a firefly luciferase marker, which enables sensitive detection of replicon suppression. The HCV replicon cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 292 μg/mL L-glutamine, 1% non-essential amino acids and 250 μg/mL G418. Cells were maintained aseptically in incubators with a 5% CO_2 atmosphere at 37 °C and were routinely passaged (2-3 times a week). The maximum passage number for cells used in the experiments was 30. The cells were tested for mycoplasma and found to be free of mycoplasma contamination.

The baby hamster kidney (BHK-21) cell line carrying a CHIKV replicon (BHK-CHIKV-NCT) was kindly provided by Prof. Andres Merits (University of Tartu, EE) and used as a model to study CHIKV replication inhibition similarly as in Pohjala et al. (2011) and Lillsunde et al. (2014).[20-21]

2.4. Evaluation of viral replicon suppression in primary screen

The HCV replicon assay was optimized and validated by dose-response experiments for the positive control, ribavirin (Sigma-Aldrich Co., Saint Louis, MO, US), which in the 24-hour experiments had an average IC_{50} value of 110 μM. In primary screening of the compound library, inhibition of the HCV replicon was evaluated after 24 h exposure to compounds. The HCV replicon cells were seeded onto opaque-white and clear-bottomed 96-well microplates (PerkinElmer Inc., Waltham, MA, US) at a density of 30 000 cells/well. The plates were incubated at 37 °C overnight. Compounds were dissolved in DMSO and before addition to plates diluted using assay medium composed of Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 292 μg/mL L-glutamine, and 1% non-essential amino acids. After 24 hours, the replicon inhibition was evaluated by measuring luciferase expression by using a Luciferase Assay System kit (Promega Co., Madison, WI, US) according to the manufacturer's instructions. A Varioskan Flash plate reader (Thermo Fisher Scientific, FI) was used for the luminescence measurements.

The CHIKV assay was carried out as previously described in Pohjala et al. (2011) and Lillsunde et al. (2014).[20-21] The positive control, 6-azauridine (Sigma-Aldrich Co., Saint Louis, MO, US) had an average IC_{50} of 2 μM.

2.5. Evaluation of effects on cell viability

In order to eliminate possible false positives, the hit compounds from the primary replicon screens were tested for host cell cytotoxicity in both replicon-carrying cell lines. Test conditions were identical to those of the primary anti-replicon assays. After exposure to the compounds, cell viability was determined by ATP quantitation using a CellTiter GLO® Luminescent Cell Viability Assay Kit (Promega Co., Madison, WI, US). The luminescent signal was recorded using a Varioskan Flash plate

reader (Thermo Fisher Scientific, FI). The cytotoxic agent polymyxin B (Sigma-Aldrich Co., Saint Louis, MO, US) was used as positive control on each assay plate and for assay validation in the cytotoxicity assays for both cell lines. Polymyxin B had a CC_{50} value of 5900 IU/mL in the CHIKV replicon cells (48-h exposure) and 7900 IU/mL in the HCV replicon cells (24-h exposure).

2.6. Dose-response experiments

Further experiments were performed for confirmed hits from the preliminary screen to evaluate dose-response with regard to both replicon inhibition as well as host cell toxicity. Dose-response experiments were carried out according to the same protocol as the primary screening, except that a 48-h incubation period was applied also for HCV replicon cells. The assay time was extended from the original 24-h incubation in the HCV model, in order to better detect potential toxic effects and obtain results comparable to the 48-h assay in CHIKV replicon cells. A minimum of three independent dose-response experiments with three replicates for each concentration were performed for each compound. Logarithmic concentration ranges were applied to all dose-response experiments and concentrations were selected to cover the full range of compound activity. A second positive control, the characterized Hsp90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (InvivoGen, San Diego, CA, US) was included in the dose-response experiments in order to enable comparison of test compounds to a known Hsp90 inhibitor. In HCV replicon cells, 17-DMAG had an IC_{50} value of 0.06 μM and a CC_{50} value of 1.7 μM [selectivity index (SI) 28]. In CHIKV replicon cells, the IC_{50} value of 17-DMAG was 0.04 μM and the CC_{50} value 0.14 μM (SI 4).

2.7. Data analysis and assay quality

The percentage of inhibition of the viral replicons was calculated by comparing the sample signal to the yielded maximum signal (DMSO vehicle in assay medium). The cytotoxic effect was calculated as a percentage by using the maximum signal (DMSO-treated cells) as the reference and wells with reagent only as the background value. For dose-response data, IC_{50} and CC_{50} values were calculated by fitting the dose-response data to sigmoidal dose-response curves using OriginPro 8.6 software (OriginLab, Northampton, MA, US). All IC_{50} and CC_{50} values are given as the average ± standard deviation of three independent experiments. Selectivity indices (SI) were calculated by dividing the CC_{50} value with the IC_{50} value. Assay quality was monitored throughout experiments by calculating the signal-to-background ratio (S/B), signal-to-noise ratio (S/N) and Z'-factor for the plates.[22]

2.8. Molecular modelling

Three-dimensional models of compounds **1-4** were built in ChemBio3D Ultra 13.0 (GAMESS interface, ChemBio3D Ultra 13.0, ChemBioOffice Ultra 13.0, CambridgeSoft, Cambridge, MA, US). Their geometries were optimized using MMFF94 force field and partial atomic charges were added.[23] Energy was minimized until the gradient value was smaller than 0.001 kcal/(mol Å). The optimized structure was further refined with GAMESS interface in ChemBio3D Ultra 13.0 using the semiempirical AM1 method, QA optimization algorithm and Gasteiger Hückel charges for all atoms for 100 steps (GAMESS interface, ChemBio3D Ultra 13.0, ChemBioOffice Ultra 13.0, CambridgeSoft, Cambridge, MA, US). Molecular docking calculations were performed using FlexX [24-25], as available in Lead IT (version 2.1.3., BioSolve IT GmbH, DE), running on four octal core AMD Opteron CPU processors, 16 GB RAM, two 750 GB hard drives, running 64-bit Scientific Linux 6.0. The receptor was prepared in a LeadIT graphical user interface using the Receptor wizard. Amino acid residues within a radius of 6.5 Å around the ligand from the X-ray structure of the human Hsp90β (PDB entry:

3NMQ[26]) were defined as the binding site. Hydrogen atoms were added to the binding site residues and correct tautomers and protonation states were assigned. Water molecules, except HOH242, and the ligand were deleted from the crystal structure.

The FlexX molecular docking program, as available in LeadIT (version 2.1.3., BioSolve IT GmbH, DE), was used for ligand docking. A hybrid algorithm (enthalpy and entropy driven ligand binding) was used to place the 'base fragment'. The maximum number of solutions per iteration and the maximum number of solutions per fragmentation parameter values were increased to 1000, while other parameters were set at their default values. Proposed binding modes and scoring function scores of the top ten highest scored docking poses per ligand were evaluated and the highest ranked binding pose was used for graphical representation in PyMOL (Delano Scientific LLC, San Francisco, CA, US).

2.9. Hsp90 binding studies by microscale thermophoresis

The compounds that exhibited dose-dependent inhibition in the HCV replicon screen were studied using microscale thermophoresis (MST) to assess binding to Hsp90. Human heat shock protein 90β (GenBank Accession No. AY359878), full length with C-terminal His tag, MW = 83 kDa, expressed in an *E. coli* expression system, was purchased from Sigma-Aldrich Co. (Saint Louis, MO, US). The protein was labelled with red fluorescent dye NT-647-NHS, by using the Monolith NT™ Protein Labeling Kit RED – NHS (NanoTemper Technologies GmbH, München, DE) according to the manufacturer's instructions. Compounds were dissolved in ethanol and a dilution series of the compounds (16 concentrations, 0.003 to 100 μM) and the control 17-DMAG (14 concentrations, 0.0015 to 12.5 μM) were prepared using the MST buffer (50 mM Tris-HCl pH 7.4 with 150 mM NaCl, 10 mM $MgCl_2$ and 0.05% Tween-20). The buffer additionally contained 5% (v/v) ethanol. The test

compounds in buffer were carefully mixed with labelled Hsp90 (final concentration 35 nM) and transferred into Monolith NT.115 premium-coated capillaries (NanoTemper Technologies GmbH, München, DE). Measurements were performed in a NanoTemper Monolith NT.115 instrument (NanoTemper Technologies GmbH, München, DE) using 20% LED power and 20% MST power. Three independent measurements were performed for each compound. Results were analyzed and the dissociation constant K_d ± standard deviation calculated based on the thermophoresis and temperature jump data from three individual experiments by using the NT Analysis software (NanoTemper Technologies GmbH, München, DE).

3. Results

3.1.Primary screening and hit selection

In primary screening of the library of 157 clathrodin and oroidin analogues, 30 compounds showed over 50% inhibition of the HCV replicon (hit rate 19%) and 15 compounds inhibited the CHIKV replicon by more than 50% (hit rate 9.6%). The full primary data for the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole structural class of oroidin analogues (**1-38**) can be found in Supplementary table S1. Most of the primary hits were excluded based on results from the confirmatory follow-up studies due to host cell toxicity (Supplementary table S1), lack of efficacy or insufficient dose-response correlation in the follow-up studies. As a result of hit evaluation, four compounds (**1**-**4**, Fig. 2) were found to show potent inhibition of the HCV replicon at low micromolar concentrations (IC_{50} value range 1.6-4.6 μM).

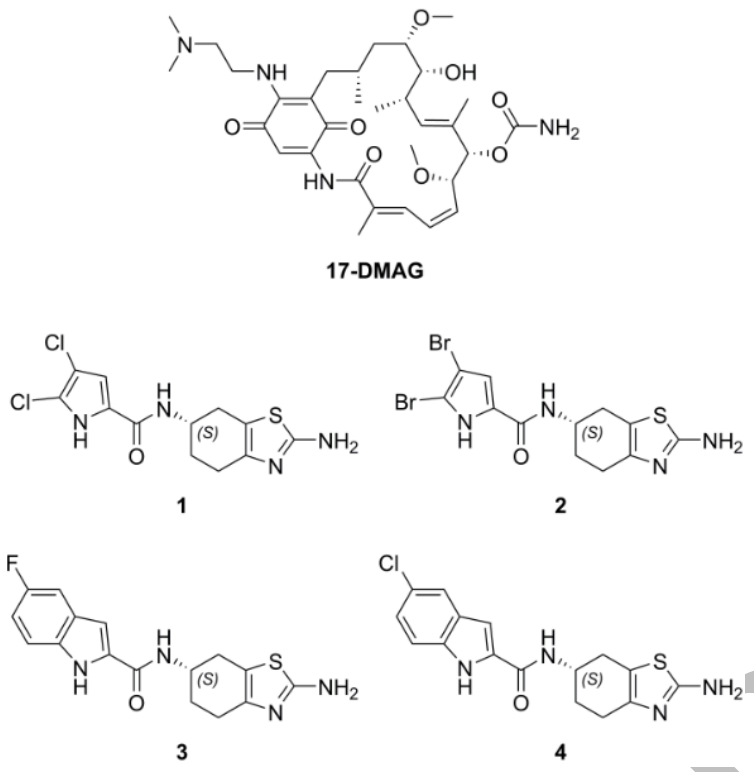

Fig. 2. Structures of compounds **1-4** and 17-DMAG, the characterized Hsp90 inhibitor used as positive control in the study.

The compounds' target selectivity was evaluated by comparing the IC_{50} values to corresponding CC_{50} values (50% cytotoxicity). The dose-response curves from anti-replicon assays and cytotoxicity experiments in HCV replicon cells are shown in Supplementary figure F1. With selectivity indices ranging from 22 up to 61 (Table 1), the compounds were shown to possess excellent selectivity. Compounds **1**-**4** were consequently chosen for further evaluation in order to elucidate their mechanism of action.

Table 1. Biological activity of compounds **1**-**4** and the reference compounds 17-DMAG and ribavirin determined in the HCV replicon system and by microscale thermophoresis. Results of the HCV replicon assay (time of exposure 48 h) are average ± s.d. from three independent dose-response

experiments with three replicates for each concentration. Dissociation constants (K_d) of the control compound 17-DMAG, compounds **1**-**4** and human Hsp90 were determined by microscale thermophoresis and are expressed as average ± s.d. of three independent experiments.

Compound	**IC_{50} HCV replicon (μM)**	**CC_{50} HCV replicon (μM)**	**Selectivity index**	**K_d Hsp90 (μM)**
1	4.6 ± 0.43	101 ± 7.9	22	19 ± 5.7
2	1.6 ± 0.08	98 ± 8.7	61	39 ± 14
3	3.1 ± 0.8	84 ± 1.8	27	79 ± 17
4	2.8 ± 0.35	78 ± 4.0	28	18 ± 3.4
17-DMAG	0.06 ± 0.01	1.7±0.25	28	0.27 ± 0.02
Ribavirin	64±7.0	> 300	ND	ND

3.2. Molecular target and selectivity

A common structural feature of compounds **1-4** is the unsubstituted 2-amino group on a 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole skeleton and a hydrophobic, substituted pyrrolamide or indolamide moiety. In contrast, compounds with unsubstituted pyrrolamide or indolamide moiety and/or substituted 2-amino group were found to be less potent or inactive. As the active compounds **1-4** had been initially found to bind to the ATP-binding site of bacterial DNA gyrase,[11] we speculated that binding to the structurally related ATP-binding pocket of the cellular chaperone Hsp90 might be the mechanism causing the observed virus replicon suppression.

To assess the assumed mechanism of action, the water-soluble Hsp90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) was evaluated in the HCV replicon cell model as an additional reference compound. Indeed, 17-DMAG very efficiently suppressed the HCV replicon (IC_{50} = 0.06 μM) whereas the suppression by compounds **1**-**4** was ca. 25- to 75-times weaker (Table 1). The impact of Hsp90 suppression on HCV in replicon cells and humanized liver mice has previously been shown by Nakagawa et al.(2007).[27] However, 17-DMAG is cytotoxic at low concentrations (CC_{50} = 1.7 μM), with a selectivity index of 28. Out of hit compounds **1-4**, the highest selectivity was observed for compound **2** (SI 61), which had 2-fold higher selectivity than the positive control 17-DMAG. The compounds data was also compared to ribavirin, the antiviral agent that has traditionally been used in clinical HCV treatment. The inhibitory effect of ribavirin in the HCV replicon model is very modest (IC_{50} 64 μM) compared to compounds **1**-**4** and 17-DMAG (Table 1).

The compounds identified as potential CHIKV inhibitors in the primary screen exhibited moderate selectivity and efficacy against the CHIKV replicon in dose-response and cytotoxicity studies (Supplementary table S1). Amongst the selected four hit compounds, compound **2** showed replicon suppression in both HCV and CHIKV models. Compound **2** inhibited the CHIKV replicon with an IC_{50} value of 42 μM and its cytotoxicity CC_{50} value was 81 μM, and it thus possessed limited selectivity and potency. Interestingly, also the control compound 17-DMAG has restricted anti-replicon selectivity in the CHIKV cell model (IC_{50} = 0.04 μM; CC_{50} = 0.14 μM; SI 4).

3.3. Investigation of the binding mode to Hsp90 by molecular docking

Bacterial DNA gyrase and human Hsp90 belong to the GHKL (gyrase, Hsp90, histidine kinase and DNA mismatch repair protein MutL) ATPase/kinase superfamily and share the unconventional Bergerat ATP-binding fold.[28] This led to suggesting Hsp90 as the possible target of the ATP-competitive DNA gyrase inhibitors **1-4**. Furthermore, the co-crystal structure of *E. coli* DNA gyrase in complex with a pyrrolamide inhibitor shows a similar hydrogen bonding network between the pyrrolamide moiety and Asp73,[12] which is also predicted by molecular docking of compounds **1-4** in the DNA gyrase ATP-binding site[11]. Therefore, plausible binding modes of compounds **1-4** in the ATP-binding site of human Hsp90 were studied by molecular docking experiments. Pyrrolamide moieties of **1** and **2** and indolamide moieties of **3** and **4** mimic the binding of the adenine ring of ATP by forming hydrogen bonds with Asp93 and Thr184 side chains and a conserved water molecule (Fig. 3). An additional hydrogen bond between Asp102 side chain and amino group of aminothiazole of **1-4** is predicted (Fig. 3). Since similar binding modes and scoring function scores of compounds **1-4** were predicted, the observed differences in experimental K_d values cannot be rationalized based on docking results.

a) b)

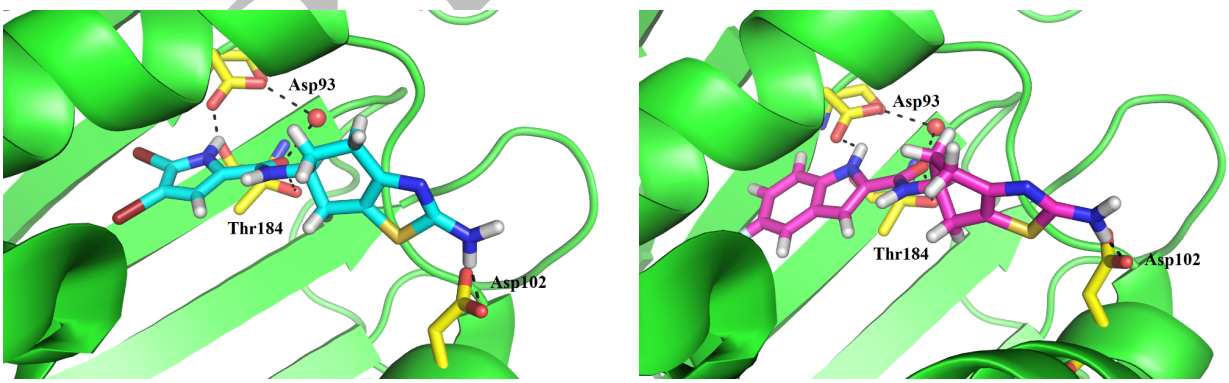

Fig. 3. Docking binding mode of compound a) **2** (in *cyan* sticks) and b) **3** (in *magenta* sticks) in the ATP-binding site of human Hsp90β (PDB entry: 3NMQ; in *green*). Amino acid residues Asp93,

Asp102 and Thr184, which are involved in formation of hydrogen bonds (*black* dashed lines) with the ligand are presented as *yellow* sticks.

3.4. Evaluation of binding to Hsp90 by microscale thermophoresis (MST)

In order to assess our hypothesis of Hsp90 as the molecular target and confirm the observations from Hsp90 docking studies, we evaluated the binding of compounds **1**-**4** to Hsp90 by microscale thermophoresis (MST), which allows determination of binding affinities based on movement of the particles on a microscopic temperature gradient.[29] Because expression of the β-isoform of Hsp90 is upregulated in HCV-infected cells, we chose to use Hsp90β in the MST experiments.[30] As in the cell-based replicon assays, the known Hsp90 inhibitor 17-DMAG was used as positive control in MST. The K_d obtained in our experiment for 17-DMAG was 0.27 ± 0.02μM, which corresponds well to the dissociation constant 0.35 ± 0.04 μM previously reported for wild-type Hsp90 by Onuoha et al. (2007).[31]

The strongest interaction with Hsp90 was observed for compound **4**, which had a K_d of 18 ± 3.4 μM (Table 1). However, the assay setup in the MST experiments allowed testing at maximum 100 μM concentration, and thus the results should be considered as a primary finding.

4. Discussion

The similarities of ATP-binding sites of DNA gyrase and Hsp90 provides a coherent explanation to why some compounds designed to bind to the ATP-binding site of bacterial DNA gyrase also interact with Hsp90. Our results are in line with previous findings, where inhibition of Hsp90 has been discovered to be an efficient pathway of interfering with virus replication. In literature, a few former

studies of Hsp90 inhibitors with anti-HCV activity have been reported.[32-34] These previous findings focused on geldanamycin and its derivatives. Although these geldanamycin derivatives were shown to be very potent Hsp90 inhibitors as well as antiviral agents, compounds **1-4** described in our study represent not only a new structural class of potential Hsp90 inhibitors, but are also small in size, synthetically easily accessible and offer great prospects for further optimization towards more potent compounds.

All of the four most promising compounds **1**-**4** identified in the replicon screens showed binding to Hsp90 in the MST experiment but there was, however, no clear correlation between the IC_{50} values in the HCV replicon assay and the binding to Hsp90 (Table 1). The compounds' IC_{50} values differ little from each other whilst there are clearer differences in cytotoxicity towards the HCV replicon host cells and Hsp90 binding. The lack of correlation between HCV replicon IC_{50} and binding to Hsp90 might be explained by several factors. The solvent in the cell-based assays was DMSO, whereas ethanol was the most suitable solvent for MST experiments. Even though the compounds were soluble in both DMSO and ethanol at the highest used concentrations, it is possible that differences in solubility behavior have affected the results. At this phase the possibility of other targets in addition to Hsp90 can also not be excluded.

The cellular functions and targets of Hsp90 are numerous. Hsp90-type proteins are encountered in virtually all cell compartments in eukaryotic cells, and they are one of the most abundant protein types found in cytoplasm.[35] Hsp90 is needed both under normal physiological conditions and under conditions of cellular stress, such as elevated temperature.[36] Hsp90 gains energy from binding and hydrolyzing ATP to assist conformational changes of more than 20 co-chaperones. Hydrolyzing ATP

causes a change in the conformation of Hsp90, which induces conformational changes in the client protein. The first identified functions of Hsp90 were facilitation of the maturation of protein kinases and steroid hormone receptors.[37-38] Other identified clients of Hsp90 are proteins involved in transcription, translation, mitochondrial function, kinetochore assembly, centrosome and cell cycle function.[36] Hsp90 targets also include proteins involved in regulation of secretory pathway functions and membrane trafficking.

Considering the important role of Hsp90 in regulation of the cellular functions and cell proliferation, it is not surprising that Hsp90 has been studied extensively as a target protein for cancer treatment. Hsp90 enables proper protein stability and folding in cancer cells, which makes tumor cells hypersensitive to Hsp90 inhibition.[39] Small molecules targeted to bind to Hsp90 are capable of disturbing the activity of receptors, kinases and transcription factors involved in oncogenesis. More strikingly, however, also viruses are almost universally dependent on Hsp90.[36] In our study, the replicon-inhibiting activity was mostly specific for either CHIKV or HCV – only compound **2** possessed activity against both the HCV and CHIKV replicons. Hsp90 has been shown to play several important roles in the HCV replication cycle. It mediates the maturation of non-structural protein 2/3, ensures the proper folding of HCV replication proteins and regulates phosphorylation of non-structural protein 5A; all of which are crucial functions for successful HCV propagation.[40] Based on current literature, the effects of Hsp90 in the viral replication cycle are better characterized for HCV than they are for alphaviruses such as CHIKV.

Rapid evolution of viruses causes complications when developing directly acting antivirals that target viral proteins, and it makes antiviral drug discovery as well as treatment of patients challenging from both medical and economic perspectives. On the contrary, targeting host factors as a strategy in

antiviral drug development offers the possibility to obtain effects against a broader spectrum of viral pathogens, with a reduced likelihood for development of resistance. The major concern related to inhibition of a host protein is naturally unwanted side effects arising from disturbance of the normal cellular functions. However, viral replication has been shown to be sensitive to Hsp90 inhibitors at concentrations that do not affect cellular viability.[36] In accordance with the literature,[36] compounds **1**-**4** caused toxicity only at high concentrations in the cell models, and the observed specificity indicates that doses effective in inhibition of replication functions are far below those that cause relevant toxicity. It is notable that the affinity of compounds **1**-**4** to Hsp90 is not as strong as that of the positive control 17-DMAG, which still leaves room for structural optimization in order to improve binding.

5. Conclusions

In conclusion, the promising inhibitory effect of compounds **1-4** in the HCV replicon system led us to investigate Hsp90 as a target behind their effects on the replication of viral RNA. Preliminary binding studies by microscale thermophoresis demonstrated binding of **1**-**4** to Hsp90 and highlighted these compounds as a potential novel structural class of Hsp90 inhibitors. The HCV replicon suppression was exhibited by these compounds at low micromolar concentrations, and most importantly, at concentrations that have no negative effects on cell viability. Questions that remain to be answered are whether the effects seen in replicon models can be replicated using full virus assays and other more advanced models. Furthermore, it remains to be determined whether the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole class of oroidin analogues are capable of inhibiting the replication of other viruses. Because of their excellent synthetic accessibility, these compounds nevertheless offer great potential for further optimization of their binding affinity to Hsp90. Inhibition of host factors such as Hsp90 may be an advantageous therapeutic strategy for treatment of viral infections in the future.

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Conflicts of interest

The authors declare no conflicts of interest.

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Supporting information

Supplementary table S1. Primary results of HCV and CHIKV replicon screens.

Supplementary figure F1. Dose-response (replicon inhibition and host cell cytotoxicity) of compounds 1-4 in HCV replicon cells.